Detecting bean golden yellow mosaic virus in bean breeding lines and in the common legume weed *Macroptilium lathyroides* in Puerto Rico[1,2]

Lydia I. Rivera-Vargas[3], Vilmaris Bracero-Acosta[4], James S. Beaver[5], Dan E. Purcifull[6], Jane E. Polston[7] and Ernest Hiebert[8]

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ABSTRACT

Bean golden yellow mosaic virus (BGYMV) is a geminivirus transmitted by whiteflies (Genus: *Bemisia*). This virus causes significant losses in common bean (*Phaseolus vulgaris* L.). Serological techniques such as enzyme-linked immunosorbent assay (ELISA) have been widely used for detection of viruses. We evaluated existing monoclonal antibodies (3F7, 2G5 and 5C5) for the detection of BGYMV isolates in bean lines in Puerto Rico. Monoclonal antibody 3F7 was the most effective in detecting the virus in tissues of line DOR 364 and susceptible cultivars Top Crop and Quest. However, it was not effective in the detection of BGYMV in lines of DOR 303, which showed typical symptoms. Samples from *Macroptilium lathyroides*, a weed that might be a possible reservoir of the virus, were also tested for viral infection. ELISA tests were inconclusive for detection of geminiviruses in *M. lathyroides*. Polymerase Chain Reaction (PCR) was also used to complement BGYMV diagnosis in *M. lathyroides* and in bean lines that showed symptoms but were negative for the ELISA test. Two sets of primers, specific for Begomovirus such as BGYMV, were used in PCR experiments. Using PCR, we were able to detect the virus in the line DOR 303 and in *M. lathyroides* tissues.

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[3]Associate Professor, Dept. of Crop Protection, P.O. Box 9030, University of Puerto Rico, Mayagüez, P.R. 00681-9030.

[4]Former Graduate Student, Dept. of Crop Protection, P.O. Box 9030, University of Puerto Rico, Mayagüez, P.R. 00681-9030.

[5]Researcher, Dept. of Agronomy and Soils, University of Puerto Rico, Mayagüez, P.R. 00681-9030.

[6]Professor, Dept. Plant Pathology, University of Florida, Gainesville, FL 32611-0680.

[7]Associate Professor, Dept. Plant Pathology, University of Florida, Bradenton, FL 34203.

[8]Professor, Dept. Plant Pathology, University of Florida, Gainesville, FL 32611-0680.

Key words: bean golden mosaic virus, bean golden yellow mosaic virus, geminivirus, monoclonal antibodies, PCR

RESUMEN

Detección del virus del mosaico amarillo dorado en líneas de habichuela y en la maleza común de las leguminosas *Macroptilium lathyroides* en Puerto Rico

El virus del mosaico dorado amarillo de la habichuela (BGYMV) es un geminivirus transmitido por moscas blancas (Género: *Bemisia*). Este virus causa pérdidas económicas considerables en la habichuela común (*Phaseolus vulgaris* L.). Las técnicas serológicas como ELISA, se han utilizado ampliamente en la detección de viruses. Se evaluaron tres anticuerpos monoclonales existentes (3F7, 2G5 y 5C5) en la detección del BGYMV en varias líneas de habichuela en Puerto Rico. El anticuerpo monoclonal 3F7 resultó el más efectivo en detectar el virus en tejidos de habichuela de la línea DOR 364 y los cultivares susceptibles Top Crop y Quest. Sin embargo, no fue efectivo en detectar el virus en la línea DOR 303 aún en presencia de síntomas típicos. También se evaluaron muestras de *Macroptilium lathyroides*, una maleza que puede ser reservorio del virus. Las pruebas de ELISA resultaron inconclusas en la detección de geminiviruses en *M. lathyroides*. Se utilizó la reacción en cadena de la polimerasa (PCR) para complementar el diagnóstico del BGYMV en *M. lathyroides* y líneas de habichuela con síntomas de virosis pero negativas a la prueba de ELISA. Se utilizaron dos pares de iniciadores en las pruebas de PCR, específicos para Begomovirus como el BGYMV. Utilizando PCR fue posible detectar el virus en los tejidos de la línea DOR 303 y en *M. lathyroides*.

INTRODUCTION

Geminiviruses (Genus: Begomovirus) are transmitted by whiteflies of the genus *Bemisia* (Homoptera: Aleyrodidae). Symptoms observed in infected plants are yellowing, mosaic, stunting and distortion of leaves and pods (Morales and Niessen, 1988). These viruses cause significant losses in common bean (*Phaseolus vulgaris* L.) in the Caribbean, Florida, Central America and Latin America (Blair et al., 1995; Morales, 2000). Recently, Fauquet et al. (2000) separated geminiviruses causing symptoms of golden mosaic into two groups: Bean Golden Mosaic Virus (BGMV) and Bean Golden Yellow Mosaic Virus (BGYMV). The golden-yellow mosaic terminology was first used by Bird et al. (1972) to describe very intense yellowing symptoms observed in *Phaseolus lunatus* and *P. vulgaris* in Puerto Rico. However, because of similar symptom description this terminology was not in use until recently. Results from the molecular characterization of geminivirus isolates showed that Brazilian or South American isolates differed from those of the Caribbean, Florida, and Central America (i.e., Mesoamerican) (Gilbertson et al.,1993; Rojas et al., 1993; Faria and Maxwell, 1999). Contrary to the Mesoamerican isolates, the Brazilian isolates cannot be transmitted mechanically and have different nucleotide sequences (Gilbertson et al., 1993; Morales, 2000).

BGMV and BGYMV are bipartite with two single-stranded circular genomes: DNA A and B (Harrison and Robinson, 1999). The viruses replicate and accumulate in the nuclei of companion cells associated with the phloem, but virus particles are in very low concentrations in infected tissues, thus making it difficult to detect (Harrison, 1985). Another obstacle associated with the study of BGMV and BGYMV isolates is their limited host range (Morales and Niessen, 1988).

Different techniques have been used for detection of geminiviruses in general and BGYMV, in particular. ELISA (Enzyme Linked Immunosorbent Assay) is a serological test that has proven useful as a diagnostic tool for geminiviruses. Cancino et al. (1995) developed three monoclonal antibodies (3F7, 2G5 and 5C5) to detect BGMV and BGYMV isolates from the Caribbean and Latin American regions. They found that monoclonal antibody 3F7 is a broad-spectrum antibody that reacted with geminivirus isolates from several geographical areas and also with several other geminiviruses. Monoclonal antibody 2G5 detected isolates from Puerto Rico, Guatemala and the Dominican Republic, whereas monoclonal antibody 5C5 detected only a Brazilian isolate.

Polymerase chain reaction (PCR) is another sensitive technique used to diagnose geminiviruses. It is based on the use of primers that are complementary to flanking regions of the DNA sequence of interest. The advantage of using PCR with geminiviruses is that these replicate in a circular double DNA intermediate strand that serves as template for PCR amplification (Hiebert et al., 1996). Major differences in DNA sequences and biological characteristics have separated BGMV isolates into two different types on the basis of PCR. Type I is represented by BGMV isolates from Brazil; and type II is represented by BGYMV isolates from Dominican Republic, Guatemala and Puerto Rico (Faria and Maxwell, 1999).

Compared with ELISA, PCR is a very sensitive technique to detect BGYMV, and PCR can also be used to analyze large numbers of samples. Disadvantages of PCR include high cost per sample, the need for adequate expertise, a well equipped laboratory and the occurrence of PCR inhibitory products in some plant extracts (Hiebert et al., 1996).

Furthermore, Chagas et al. (1981) discussed the possibility of various weeds as alternative hosts for crop infecting geminiviruses in Brazil. Among them *Canavalia* sp., *Crotalaria* spp., *Sida* spp., *Rhynchosia* sp., *Macroptilium lathyroides* and *M. erythroloma* were described as natural and experimental hosts of the geminivirus. The same authors found *M. erythroloma* exhibiting golden mosaic symptoms associated with whiteflies (*B. tabaci*) and suggested the possibility that this species might serve as a reservoir of BGYMV in nature.

The main objective of this investigation was to compare and evaluate the effectiveness of existing monoclonal and polyclonal antibodies in detecting and characterizing BGYMV in Puerto Rico. The application of this research involved ELISA to evaluate bean lines for resistance to BGYMV. We used PCR primarily to complement ELISA in detecting the presence of BGYMV in plant tissues. In addition, we attempted to clarify the role of the common legume weed *Macroptilium lathyroides* as a possible alternative host for the virus in Puerto Rico.

MATERIALS AND METHODS

Plant material: Bean lines with different genes resistant to BGYMV, such as DOR 482, DOR 364 and DOR 303, were used to characterize reactions to the virus. Snap bean cultivars Quest and Top Crop were used as positive controls because of their susceptibility to BGYMV and typical symptom expression. Three seeds of each bean line were planted in Pro-mix®[9] in 15-cm-diameter pots and grown in a greenhouse with a mean temperature of 27°C and a relative humidity of 75%.

BGYMV Inoculation: Bean plants were inoculated with viruliferous whiteflies taken from BGYMV-infected plants as described by Adames-Mora et al. (1996). Virus inoculum was obtained from an established colony of viruliferous whiteflies kept on susceptible cultivar Top Crop under greenhouse conditions. Twelve viruliferous whiteflies were allowed to feed for 72 h in a cage containing three bean plants. Non-inoculated plants were used as controls and were kept in a separate greenhouse. The experimental unit was a pot containing three plants of the materials to be tested. A complete randomized design with three replications was used. Five days after inoculation, a trifoliate from each of the inoculated plants and from control plants were taken for ELISA tests. Additional samples were taken every five days until 30 days after inoculation. Plants were periodically examined for symptom expression.

ELISA assays: Monoclonal antibodies (MAb) 3F7, 2G5 and 5C5, developed by Cancino et al. (1995) were used for this evaluation. Microplate wells were coated with polyclonal antiserum #1110 prepared from purified *M. lathyroides* geminivirus at 1.3:1,000 dilution of coating buffer (100 µl per well). Monoclonal and polyclonal antibodies were provided by the Hybridoma Core Facility of the University of Florida at Gainesville, FL. Polyclonal antibody coated plates were incubated for

[9]Trade names in this publication are used only to provide specific information. Mention of a trade name does not constitute a warranty of equipment or materials by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

1 h at room temperature, and then washed three times with phosphate buffer containing Tween-20 (PBST).

Antigen samples were prepared by pressing a trifoliate using a roller press (Ravenel Specialties, Co. Seneca, SC). Plant sap was diluted 1:10 in phosphate buffer pH 7.2 (PBS). One hundred µl of sample was added to each well and replicated three times. Plates were incubated for 1 h at room temperature and washed 10 times with PBST. Monoclonal antibodies were added at 1:10,000 dilution in PBS pH 7.2 and a 100 µl sample was added to each well. Plates were incubated for 1 h at room temperature and washed six times with PBST. One hundred µl of a 1:2,000 dilution of antimouse IgG, conjugated with alkaline phosphatase (Sigma, Saint Louis, MO) in PBS at pH 7.2 was added to each well. Plates were incubated for 1 h at room temperature and were washed three times with PBST. P-nitrophenylphosphate (PNPP) was added at 1 mg/ml of substrate buffer (pH 9.6) before use in the wells, and 100 µl of PNPP was added to each well.

Absorbance due to color changes in plate wells was read at 405 nm, 30 min and 1 h after incubation by using an ELISA plate reader (Dynatech MR 5000, Dynatech Laboratories Inc., Chantilly, VA). PBS was used as control. Samples from virus infected susceptible bean cultivars were used as positive controls.

Polymerase Chain Reaction: We used the methodology developed by Dellaporta et al. (1983) for viral DNA extraction. Two sets of primers were used for PCR, primers for viral DNA component A were PAL1v1978/ PAR1c496; for component B, PBL1v2040/PCRc1 (Table 1). The PCR reaction mixture (100 µl) consisted of 20 to 40 µl of sample DNA, dNTPs and primer concentrations of 25 µM for each nucleotide and 0.2 µM for each primer. Magnesium concentration was between 0.5 and 2.5 mM. Taq polymerase (Promega, Madison, WI) was used according to manufacturer's instructions. Viral DNA was amplified with a Thermocycler (Perkin Elmer, Norwalk, CT) using 30 cycles of melting, annealing and DNA extension conditions for 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C. Amplified DNA fragments were electrophoresed at 3 V/cm for 5 h in 0.7% agarose (Rojas et al., 1993) in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8). DNA bands were visualized with ultraviolet light after staining in ethidium bromide and photographed (Polaroid camera, Model 617572; Polaroid film 667). A 1-kb ladder size marker Lambda DNA/EcoRI = Hind III (Promega Corp., Madison, WI) was used for DNA fragment size comparison.

Macroptilium lathyroides: An experiment was conducted with *Macroptilium lathyroides* to evaluate its role as a possible BGYMV reservoir. Seeds were scarified by cutting one of the tips and placing the seed in standard size petri plates in moist chambers. After germina-

TABLE 1.—*DNA sequences of nucleotide primers used for PCR.*

Primer	Nucleotide sequence[1]
PAL1v1978	5' GCATCTGCAGGCCCACATYGTCTTYCCNGT 3'
PCRc1	5' CTAGCTGCAGCATATTTACRARWATGCCA 3'
PAR1c496	5' AATACTGCAGGGCTTYCTRTACATRGG 3'
PBL1v2040	5' GCCTCTGCAGCARTGRTCKATCTTCATACA 3'

[1]Nucleotides at degenerate positions are represented by single letter (IUPAC ambiguity code), K = G,T; N = A,C,G, T; R = A, G; W = A, T; Y = C, T (Rojas et al., 1993).

tion, three seeds of *M. lathyroides* were planted as described before for bean plants. BGYMV inoculation procedures were followed as described for bean plants. Twelve days after inoculation, three young leaves from inoculated and control *M. lathyroides* plants were taken for ELISA tests. Susceptible bean cultivar Top Crop was used for comparison. ELISA and PCR procedures were followed as described for bean plants to detect BGYMV in *M. lathyroides* tissues.

A second experiment, using *M. lathyroides*, was conducted as described above, with the exception that the viruliferous flies were obtained from soybean plants [*Glycine max* (L.) Merrill] from Asgrow Seed Co. fields, located at Isabela, P.R. A whitefly colony was established in bean cultivar Top Crop under greenhouse conditions and was used as the initial source of virus inoculum. Ten viruliferous whiteflies were allowed to feed for 48 h in a cage containing three *M. lathyroides* plants. BGYMV inoculation and ELISA procedures were followed as described above.

RESULTS AND DISCUSSION

Evaluation of monoclonal antibodies in the assessment of BGYMV

Bean breeding lines: The optimal conditions for performing the ELISA occurred 20 days after inoculation. Monoclonal antibodies 3F7 and 2G5 reacted with viral particles present in susceptible line DOR 364 and cultivar Quest; 3F7 was the most effective in detecting the virus (Figure 1). Consistent with reports by Adames Mora et al. (1996), line DOR 364 did not show well-defined symptoms 10 to 12 days after inoculation. This line also exhibited lower virus titer than susceptible cultivar Quest and positive control Top Crop. The low virus titer in their tissues has proved to be of great genetic value to farmers. Under field conditions in Puerto Rico and the Dominican Republic, Blair et al. (1994) observed delayed symptom expression in DOR 364 correlated with a reduction of BGYMV incidence. The tolerance shown by this line was demonstrated by its high yield and reduced levels of disease and

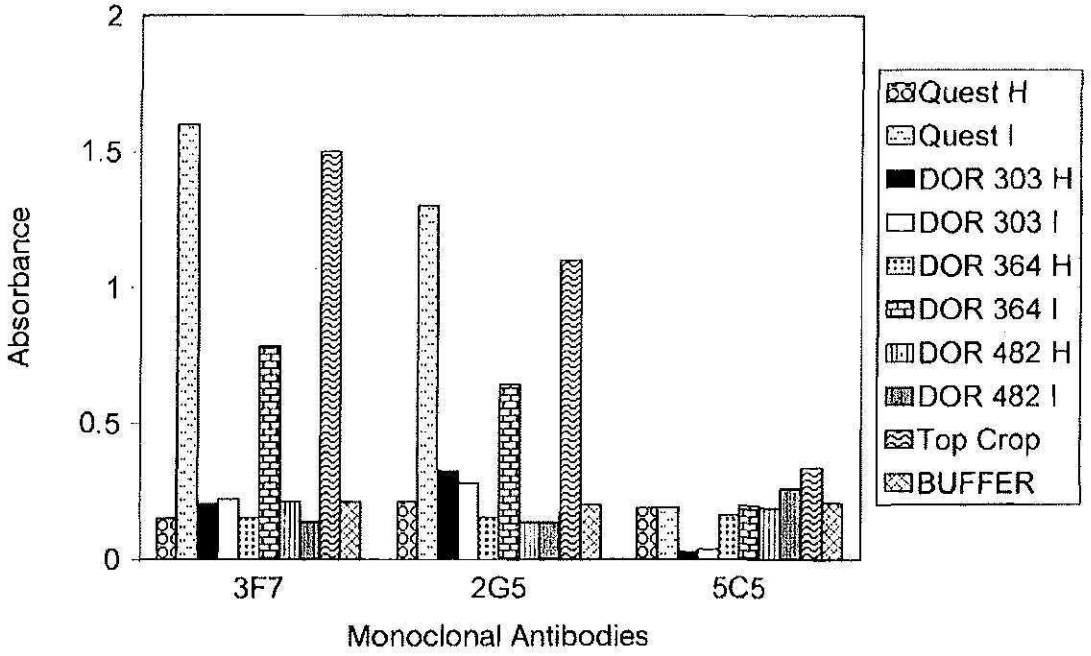

FIGURE 1. Results of ELISA tests for BGYMV in different bean lines and cultivars using three monoclonal antibodies after 1h of incubation (H = Healthy; I = infected).

spread. In other studies, this line showed satisfactory yields if infection occurred after the initiation of flowering (Morales and Niessen, 1988).

None of the monoclonal antibodies were able to detect BGYMV in cultivar DOR 303. This line did not show the typical yellow mosaic symptoms, but plants were dwarfed and pods deformed. This reaction occurred under field and greenhouse conditions with moderate to high levels of disease incidence (Vélez et al., 1998). In bean, lack of mosaic symptom expression is conditioned by recessive genes. Recessive genes such as *bgm-1* and *bgm-2* have been used as a source of partial resistance in *P. vulgaris* (Urrea et al., 1996; Vélez et al., 1998). The line DOR 303 carries a recessive resistance gene *bgm-2*, conferring protection from chlorosis induced by BGYMV infection. A recessive gene, *dwf* in DOR 303 that produces a dwarfing response to BGYMV infection, appears to provide no protection against BGYMV (Vélez et al., 1998; Morales, 2000). It is possible that a low virus titer in this line might be responsible for no reaction with MAb 3F7. Furthermore, no reaction occurred with resistant cultivar DOR 482 when screened with MAb 3F7. Our results are consistent with those obtained by Cancino et al. (1995) in which the broad spectrum MAb 3F7 was found to react efficiently in ELISA tests with extracts from plants infected with BGYMV.

Monoclonal antibody 2G5 reacted with BGYMV isolates obtained from the field in Puerto Rico, but the reactions were weaker than the reactions with MAb 3F7. Monoclonal antibody 5C5 did not react with

the BGYMV isolates used during the experiments, thus confirming the differences between isolates from Mesoamerica and Brazil.

Macroptilium lathyroides: The golden yellow mosaic symptoms caused by BGYMV were observed in *M. lathyroides* and Top Crop plants, 12 to 15 days after inoculation. In *M. lathyroides* the symptoms were observed only in leaf veins and not in all leaf areas. ELISA tests with *M. lathyroides* and Top Crop showed relatively lower readings from infected plants in samples taken 12 days after inoculation (Figure 2A) than the elevated readings obtained with MAb 3F7 from bean geminivirus-infected tissues in samples taken 20 days after inoculation (Figure 1). Also fairly high background reactions with extracts from non-infected plants and the buffer made the test inconclusive (Figure 2A). Monoclonal antibodies 2G5 and 5C5 showed a very weak reaction with all healthy and infected samples (Figure 2A).

The second experiment conducted using ELISA to detect BGYMV in *M. lathyroides* and Top Crop tissues showed that MAb 3F7 was more effective in detecting the virus in infected tissues (Figure 2B) than MAbs 2G5 and 5C5. The highest readings were observed in cultivar Top Crop after 1 h of incubation. Again, the readings were lower than those observed in infected bean lines 20 days after inoculation (Figure 1). Apparently, virus titer varies in plant tissues and is a very important factor that has to be taken into account. In addition, virus titer might differ between the two species during their infection cycles. Further studies are needed to clarify these findings.

Other investigators have reported that ELISA could not distinguish between BGMV and various weed mosaics such as *Boerhavea* mosaic, *Euphorbia* mosaic, *Jatropha* mosaic and *Rhynchosia* mosiac. They also mentioned that ELISA tests had failed to detect the viral antigen in field isolates of *Rhynshosia* mosaic, *Sida* mosaic and BGMV in *Malvastrum coromandelianum* (Haber et al., 1987). The same authors mentioned that most of the agents of the rugose mosaic were refractory to conventional virus purification and characterization and this might be the case for *M. lathyroides*. They used fingerprint diagnosis to confirm the presence of distinct sets of restriction fragments that specifically hybridized with BGMV DNA sequences in *M. lathyriodes* and *Malvastrum coromandelianum*. In Jamaica, Roye et al. (1997) reported that geminiviruses infecting weeds including *M. lathyroides* are different from those infecting important crops and defined a new geminivirus cluster.

Polymerase Chain Reaction (PCR) in the diagnosis of BGYMV

Degenerate PCR primers have been used for the amplification of components A and B of geminiviruses. The primers were designed from

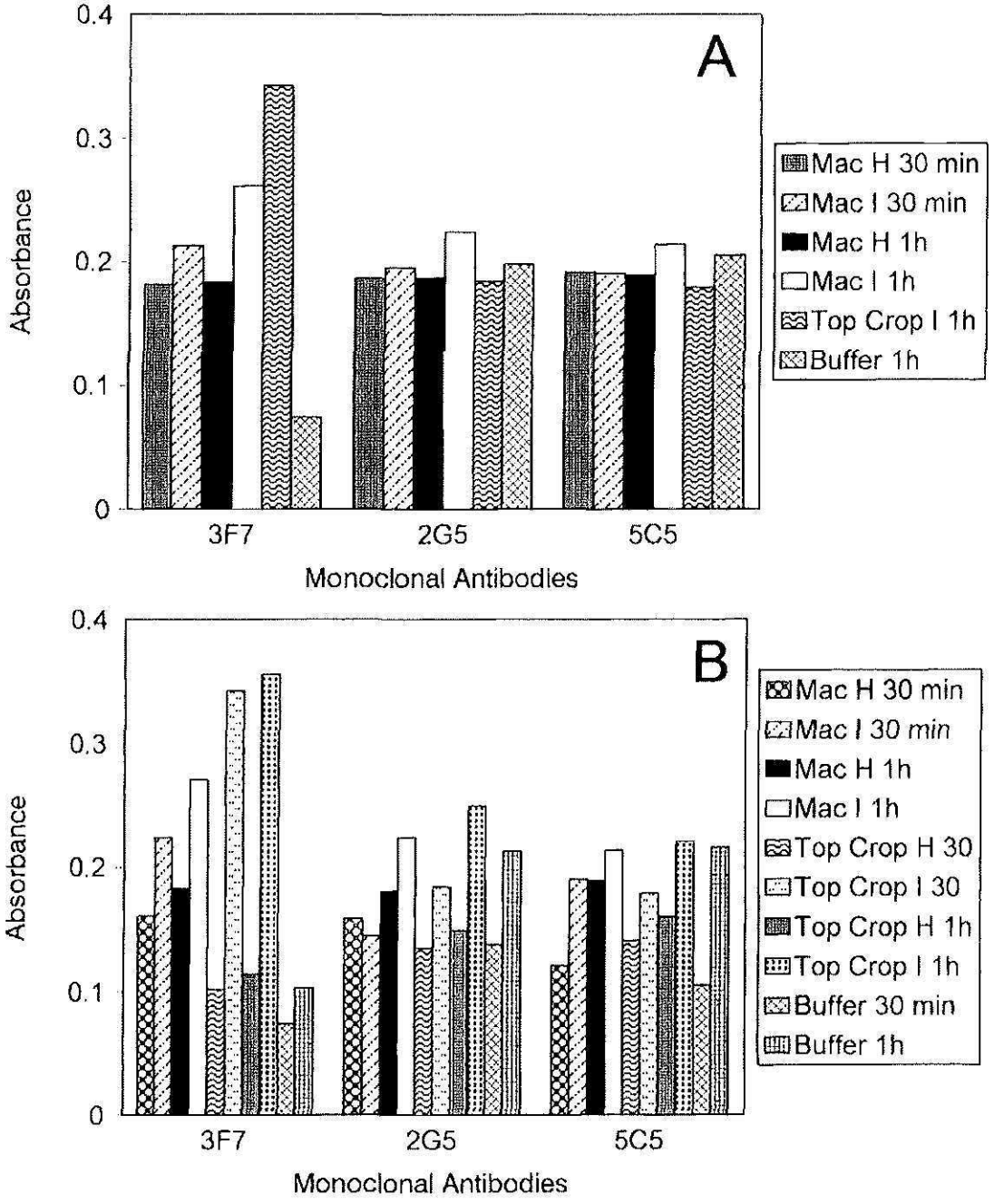

FIGURE 2. Results of ELISA tests for BGYMV in *M. lathyroides* (Mac) using three monoclonal antibodies after 30 min and 1 h of incubation (H = Healthy; I = infected). A. First experiment, B. Second experiment.

highly conserved regions of the viral genome and have been used in geminivirus diagnosis (Rojas et al., 1993). Two sets of primers were used to detect BGYMV in bean lines (DOR 303, DOR 482, DOR 364), susceptible cultivar Top Crop and *M. lathyroides*. Primers for DNA component B were more sensitive than those for component A in the detection of BGYMV in plant tissues (Figures 3 and 4). Polymerase Chain

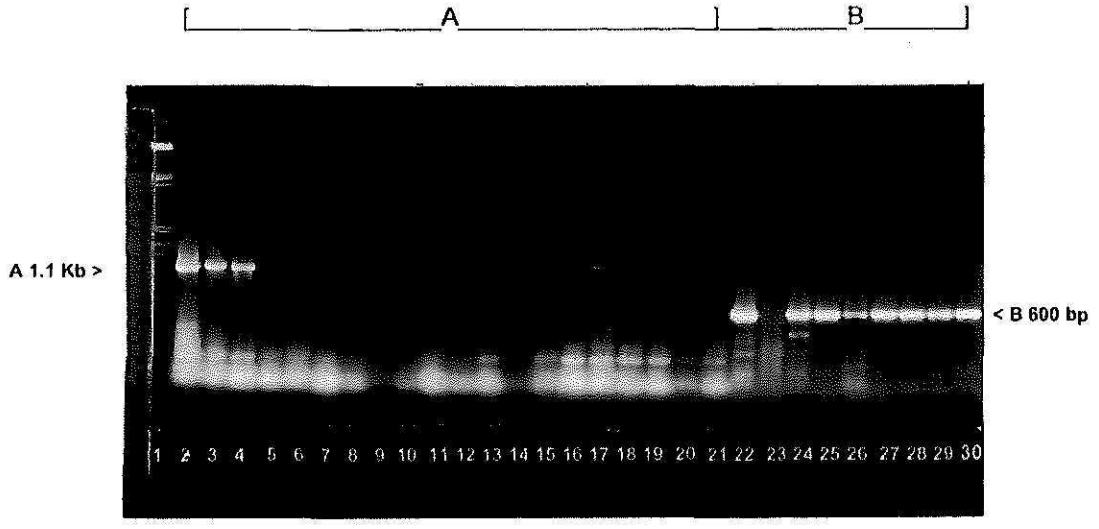

FIGURE 3. Agarose gel electrophoresis of PCR products from DNA of BGYMV. Two sets of primers were used to detect components A or B of BGYMV in different bean lines and *M. lathyroides*. Ladder size marker = 1.0 kb (lane 1); *M. lathyroides* infected (lanes 2-12); Top Crop healthy (13); Top Crop infected (14); DOR 303 healthy (15 and 16); DOR 303 infected (17); DOR 364 healthy (18); DOR 364 infected (19); DOR 482 healthy (20); DOR 482 infected (21); *M. lathyroides* infected (lanes 22 to 30).

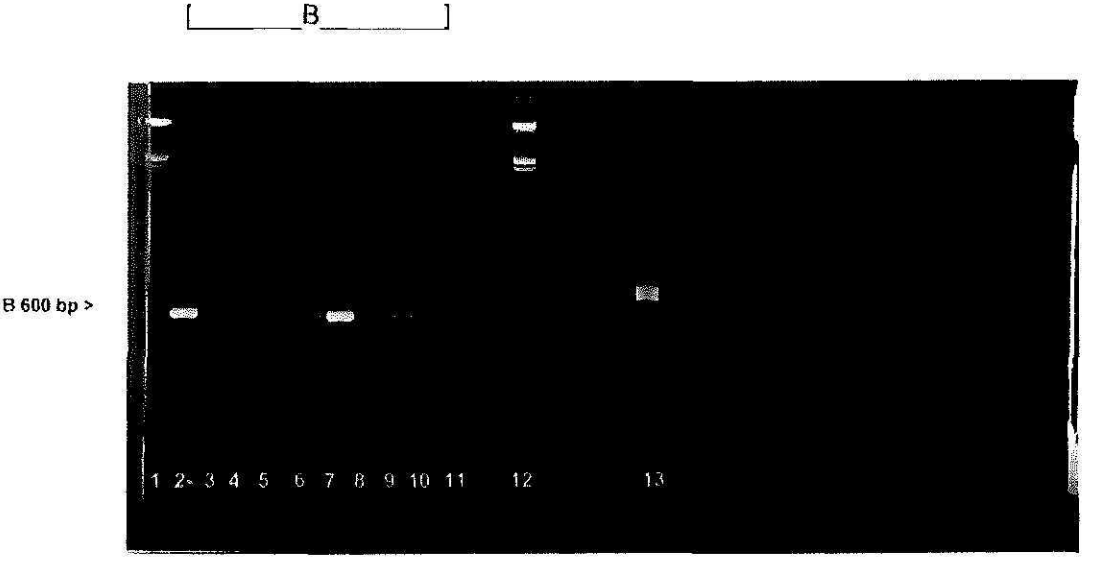

FIGURE 4. Agarose gel electrophoresis of PCR products from DNA of BGYMV. A set of primers was used to detect component B of BGYMV in different bean lines and *M. lathyroides*. Ladder size marker = 1.0 kb (lanes 1, 12 and 13); *M. lathyroides* infected (2); Top Crop healthy (3); Top Crop infected (4); DOR 303 healthy (5 and 6); DOR 303 infected (7), DOR 364 healthy (8); DOR 364 infected (9), DOR 482 healthy (10) and DOR 482 infected (11).

Reaction was sensitive enough to detect BGYMV not only in tissues of susceptible bean lines and DOR 364 but also in DOR 303 with primers for component B (Figure 3). However, primers for component B showed

that some virus contamination occurred in the greenhouse because a band at 600 bp was observed in healthy tissues of various bean lines. To date, no bean line or cultivar has proven to be totally immune to BGYMV. Apparently, low titer of viral particles can be detected in bean tissues of lines with various levels of resistance such as DOR 303 and DOR 482. Although DOR 482 showed no symptom expression, a faint band was observed by using primer for component B (Figure 4).

Primers for component A were able to detect geminivirus infections in four out of 10 *M. lathyroides* plants (Figure 3). On the other hand, primers for component B were able to detect geminivirus infections in eight out of 10 inoculated *M. lathyroides* plants (Figures 3 and 4). Through the use of primers for component B, we were able to show that the geminivirus present in plant tissues is a bipartite virus such as BGYMV. However, further experiments dealing with infectivity cycles between *M. lathyroides* and *P. vulgaris* and DNA sequencing are needed to confirm whether infections in *M. lathyroides* are related to BGYMV.

Conclusions from these results are the following: The MAb 3F7 reacted with BGYMV present in the tissues of susceptible bean cultivars and lines, and in resistant lines with delayed symptom expression. The MAb 2G5 reacted with BGYMV isolate but showed a weaker reaction than 3F7. The MAb 5C5 did not react with BGYMV isolate from Puerto Rico. ELISA tests were inconclusive for geminivirus detection in *M. lathyroides* using monoclonal antibodies. Polymerase Chain Reaction was more effective detecting BGYMV in resistant bean lines (DOR 303, DOR 482 and DOR 364), susceptible cultivar Top Crop and the weed, *M. lathyroides*. Primers from DNA component B were more sensitive than the primers from component A in the detection of BGYMV in plant tissues, including moderately resistant line DOR 303. Polymerase Chain Reaction was effective in detecting a geminivirus in *M. lathyroides* but the relationship of this geminivirus to the BGYMV in beans needs further research.

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